ISOLATION OF THE COMPLEMENTARY DNA FOR HUMAN TRANSCOBALAMIN II[1]

O. PLATICA, R. GENECZKO*, A. REGEC, E.V. QUADROS, AND S.P. ROTHENBERG[2]

Division of Hematology/Oncology, Department of Medicine and the *Department of Microbiology and Immunology, SUNY-Health Science Center and Veterans Administration Medical Center, Brooklyn, N.Y. 11203

ABSTRACT. A complementary DNA (cDNA) clone coding for transcobalamin II (TCII) has been isolated from a human umbilical vein endothelial cell cDNA library. The cDNA is 1.9 Kb and includes the nucleotide sequence which encodes the NH_2-terminal 19 amino acids of human TCII. The size of the cDNA is sufficient to code for the entire protein and also contains the nucleotide sequence coding for a 24 amino acid leader peptide and a long untranslated 3' region. The availability of this cDNA will provide the opportunity to characterize genetic disorders of TCII.

INTRODUCTION. TCII is a cobalamin (Cbl, vitamin B12) binding protein in the plasma which is essential for the cellular uptake of the vitamin (1). Congenital absence of TCII (2) or the synthesis of an abnormal protein (3), results in intracellular Cbl deficiency and megaloblastic anemia. Though these disorders have been well recognized in infants and children since 1971 (2), there have been no reported studies defining the associated genetic defect. Human TCII has recently been purified in our laboratory and the sequence of 19 amino acids comprising the NH_2-terminal domain has been determined (4). Our recent observation, that human umbilical vein endothelial cells in culture and in situ synthesize and secrete TCII (5), prompted us to screen a cDNA library from human endothelial cells for the TCII cDNA clone using an oligonucleotide probe corresponding to the NH_2-terminal amino acid sequence of the protein.

MATERIALS and METHODS. The cDNA library, prepared from cultured human umbilical vein endothelial cells and cloned into λgt 10, was kindly provided by Dr. Timothy Hla and Dr. Thomas Maciag of the Jerome Holland Laboratories for Biomedical Science, The American Red Cross, Rockville, MD. T_4 polynucleotide kinase and EcoR1 restriction endonuclease were purchased from Bethesda Research Labs (Bethesda, MD) and New England Biolabs (Beverly, MA) respectively. [^{32}P]αdCTP and [^{32}P]γATP were obtained from New England Nuclear (Boston, MA). The Geneclean Kit was purchased from BIO 101 (LaJolla, CA).

The cDNA library was screened using oligonucleotides coding from amino acids two to nine of the NH_2-terminal domain of TCII. The oligonucleotides were synthesized on a Biosearch 8600 and then purified by reverse-phase HPLC using a 10-40% acetonitrile gradient. The probe was 23 nucleotides long and consisted of a mixture of 92 oligonucleotides that were selected on the basis of human codon usage. For screening, the probe was end-labeled with T_4 polynucleotide kinase and [^{32}P]γATP to a sp act of $3x10^8$ cpm/μg. The phage library was grown in E. coli hfl at an initial density of approximately 50,000 plaques per 150 mm diameter Petri dish.

Nitrocellulose replicas of each plate were denatured and neutralized and then baked at 80°C for 2 hr according to standard procedures (6). After washing the filters in 3xsaline/sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 2 hr at 55°C, they were prehybridized at 48°C for 2 hr in 6xSSC, 1 X Denhardt's solution, 0.5% SDS, sodium pyrophosphate containing salmon sperm DNA (100 μg/ml). The hybridization was performed at 48°C for 16 hr in the same solution containing tRNA (20 ng/ml) instead of salmon sperm DNA and the radioactive probe (20 ng/ml). The filters were washed at 37°C for 1 hr and at 58°C for 23 min in 6xSSC, 0.05% sodium pyrophosphate and then exposed to Cronex R7 film for at least 18 hr. Three positive plaques were identified in the primary screening. One of the positive plaques was selected at random and plaque purified.

Approximately 3 μg of plaque purified phage DNA were prepared using the miniprep technique (7). A 1.9 Kb cDNA was identified following digestion of the phage DNA with EcoR1, agarose gel electrophoresis, Southern blotting and hybridization with the labeled oligonucleotide probe. Additional phage DNA was prepared from a 200 ml liquid culture using the standard CsCl gradient procedure (8). Following EcoR1 digestion and agarose gel electrophoresis, the 1.9 Kb insert was extracted from the gel using Geneclean Kit and the protocol provided by the manufacturer. The purified insert was then subcloned into the EcoR1 site of pUC 18 plasmid which was then used to transform the JM109 strain of E. coli. The preparation of the nitrocellulose replicas, the processing of the filters and hybridization to the oligonucleotide probe were the same as for the phage screening described above.

The plasmid DNA was amplified in liquid culture and purified by alkaline lysis and the ethidium bromide/CsCl gradient method (8).

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For sequencing, the DNA was repurified on a CsCl gradient followed by phenol extraction and ethanol precipitation. The DNA was then denatured in alkali (0.2N NaOH) and primers were annealed at 65°C for 2 min. Extension/labeling reactions were carried out with primed DNA using a master mix (3 μM dCTP, dTTP, dGTP, 0.1M DTT, 10 μCi [^{32}P]αdATP) and Klenow fragment (2.0 units) at 50°C for 5 min. Chain terminations were performed by the addition of dideoxy dNTP's and incubation at 50°C for 10 min. The reaction was stopped by adding buffer containing formamide and denatured by heating in a boiling-water bath for 2 min (9). Electrophoresis was performed in 7% acrylamide wedge gels containing urea (8M) at a constant current of 70 watts. Following electrophoresis, the gels were dried and exposed overnight to Kodak X-AR film. The sequencing primers used were standard forward and reverse universal primers designed for use with M13 phage and pUC plasmid vectors (10).

RESULTS. Fig. 1 shows the 1.9 Kb cDNA insert identified by Southern blotting of the EcoR1 digest of the clone isolated from the endothelial cell cDNA library.

The nucleotide sequence of the cDNA determined from the 5' end of the forward strand is shown in Fig. 2. Following the EcoR1 ligation site, there is a stretch of 72 nucleotides encoding a 24 residue leader peptide. This is not likely to be the full length leader peptide since there is no methionine initiation codon following the EcoR1 ligation site. The nucleotide sequence

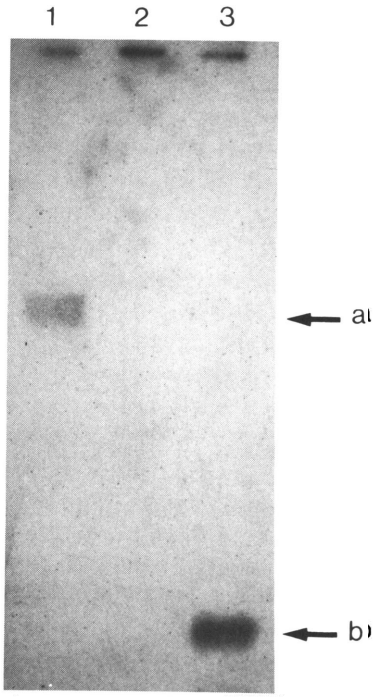

Figure 1. The Southern blot of the TCII cDNA clone. Lane 1 contains the undigested cDNA clone and Lane 3 contains the EcoR1 digest of the plaque purified cDNA clone which shows the 1.9 Kb insert. Lane 2 contains the Hind III digest of λ-phage DNA which was visualized by ethidium bromide staining of the gel prior to transfer and served as size markers as well as a negative control for the hybridization.

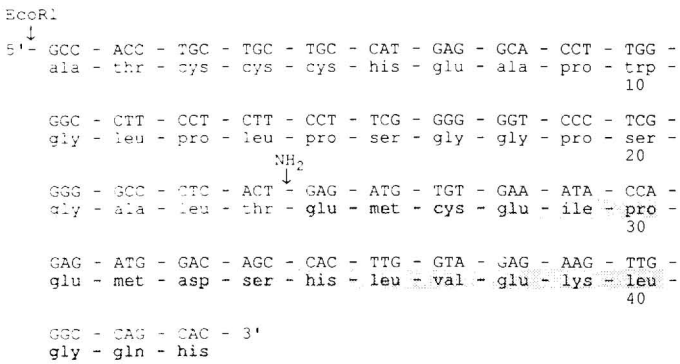

Figure 2. The nucleotide sequence and the deduced amino acid residues of the TCII cDNA. The first 72 nucleotides following the EcoR1 ligation site code for 24-amino acids of the leader peptide. The shaded area indicates the deduced amino acids that are identical to the NH_2-terminal 19 residues of TCII.

that follows, however, codes for the amino acids which are identical to the 19 NH_2-terminal amino acids obtained for TCII by direct peptide analysis (4).

DISCUSSION. The identical homology between the amino acid sequence deduced from the nucleotide sequence of the 5' end of the forward strand and the NH_2-terminal 19 amino acids determined by direct sequencing of TCII (4) provides unambiguous evidence that this clone, isolated from a human umbilical vein endothelial cell cDNA library, is the TCII cDNA. The 1.9 Kb insert contains 72 nucleotides coding for a 24 residue portion of the leader peptide and is sufficiently long to code for the entire protein containing approximatley 400 amino acids plus a long stretch of an untranslated 3' region.

An immediate advantage derived from the isolation of the TCII cDNA will be the opportunity to study the polymorphism of the protein at the genetic level (11) and the elucidation of the genetic defect(s) which are characterized by the absence of the TCII protein (2) and the synthesis of an immunoreactive but nonfunctional form of TCII (3).

In addition to these genetic studies, the complete amino acid sequence deduced from the nucleotide sequence of the cDNA will provide a more definitive characterization of the secondary and tertiary structure of TCII.

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[2]To whom reprint request should be sent.

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